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Migration of Co-cultured Endothelial Cells and Osteoblasts in Composite Hydroxyapatite/Polylactic Acid Scaffolds

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Abstract—Regeneration of bone in large segmental bone defects requires regeneration of both cortical bone and trabecular bone. A scaffold design consisting of a hydroxy apatite (HA) ring surrounding a polylactic acid (PLA) core simulates the structure of bone and provides an environment for indirect and direct co culture conditions. In this exper iment, human umbilical vein endothelial cells (EC) and normal human primary osteoblasts (OB) were co cultured to evaluate cell migration and interactions within this biphasic composite scaffold. Both cell types were able to migrate between the different material phases of the scaffold. It was also observed that OB migration increased when they were co cultured with ECs, whereas EC migration decreased in co culture. The results show that co culture of ECs and OBs in this composite biphasic scaffold allows for migration of cells throughout the scaffold and that pre seeding a scaffold with ECs can increase OB infiltration into desired areas of the scaffold.

Keywords—Endothelial cell migration, Osteoblast migration, Ceramic scaffold, Polymer scaffold, Bone regeneration.

INTRODUCTION

Extremity injuries are prevalent types of injury in civilian trauma and modern warfare, as exemplified in the current wars in Iraq and Afghanistan.[21] Large segmental bone defects as a result of trauma are especially troublesome. Extensive bone debridement, chronic infection, and non-union due to these injuries result in significant morbidity. These defects are difficult to treat, and amputation is recommended for defects larger than 10 cm.[8] Autologous posterior iliac bone grafts are currently the gold standard treatment for large segmental defects, but this treatment is limited by donor site morbidity and the small amount of bone that is available for harvesting. Each posterior iliac has enough tissue to graft 4 cm of tibia or 8 total cm per person.[11] Other treatment options include vascularized free tissue transfers and the Ilizarov technique, but these two techniques have variable results, long healing times, and significant donor site morbidity.[8]

Many tissue-engineering strategies for bone regeneration utilize combinations of scaffolds, cells, and growth factors, and attempts to regenerate mineralized bone matrix have had moderate results.[9] More recently, the focus has shifted to establishing vasculature in the area of regeneration because good vascularization is necessary for the regeneration of bone. Studies have shown improved osteogenesis *in vivo* through the use of endothelial cell (EC) and osteoblast (OB) co-cultures in scaffolds.[32,33]

When segmental bone defects are healed naturally, cortical and trabecular bone are formed, along with vasculature to support them.[8] In current treatment methods for segmental bone defects, avascular scar tissue is removed to expose the vasculature or small holes are drilled in surrounding bone before the placement of the grafts in order to encourage vascular ingrowth.[8] These methods work for smaller defects, but unfortunately, there is no current treatment modality that can reliably heal large defects.

Differentiation of cells occurs in response to local biomechanical conditions.[19] Unfortunately, most patients with large defects are unable to apply the appropriate loads needed to initiate this differentiation to the injured areas. A method to circumvent this

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problem is to create an environment utilizing materials with mechanical properties that help cells differentiate to the appropriate structure and function. To accomplish this, a biphasic scaffold modeled after the natural structure and function of bone has been developed. This scaffold is composed of a hydroxyapatite (HA) ring and an oxygen gas–plasma (GP)-treated polylactic acid (PLA) core.

Polylactic acid is a biodegradable polymer widely used for tissue-engineering scaffolds and other *in vivo* applications.[2,16] Oxygen gas–plasma-treated PLA scaffolds were chosen for this application because they have been shown to enhance angiogenesis *in vivo* in subcutaneous mouse models compared to non-treated PLA scaffolds.[22]

Hydroxyapatite (HA) scaffolds similar to the ring used in this scaffold design have been shown to support bone and vessel formation *in vivo* in a canine model.[3] This scaffold's porosity is 85% with an average pore size of 500 μm.[12] The pore size of calcium phosphate scaffolds is an important factor for vascularization and osseointegration. Although smaller pore sizes have more surface area, pore sizes greater than 210 μm have been found to have increased amounts of bone and vessel formation than scaffolds with smaller pores.[17] It is believed that the configuration of materials with different mechanical properties and the gas–plasma-treated inner core will enhance vascular network formation in the middle of the scaffold while supporting the formation of bone on the periphery.

In addition to utilizing a scaffold that more closely replicates natural bone, it is also important to create a cell environment that more closely replicates the cellular processes that occurs during the natural bone healing, where multiple cells types migrate into the wound area and interact with each other through various paracrine and juxtacrine pathways and intercellular contacts.[7]

Since the goal of bone tissue engineering is to produce vascularized bone tissue, it is important to study EC and OB co-cultures in a scaffold rather than only studying cell monocultures. In existing co-culture studies which use scaffolds, migration patterns of the cells within the scaffolds have not been investigated.[6,23,24,28] It is unknown if the cells have a preferred direction of migration or if the type of material on which the cells are seeded affects the migration patterns.

The goal of this study was to evaluate the migration of co-cultured ECs and OBs in a HA and PLA biphasic tissue-engineering scaffold. It was hypothesized that ECs and OBs co-cultured together will interact with each other and alter the cell migration patterns compared to when they are cultured alone. Enhancing our understanding of EC and OB co-cultures is important to the eventual goal of developing a scaffold which supports cortical bone growth on the outside and vasculature formation on the inside for the repair of large segmental defects in bone.

MATERIALS AND METHODS

Cell Culture

Human primary OBs were purchased from Lonza (Allendale, NJ) and cultured in OB growth media containing ascorbic acid and antibiotics (Lonza, Allendale, NJ). All cells were used on first or second passage. Human umbilical vein ECs (HUVEC) were purchased from Invitrogen (Carlsbad, CA) and cultured in Lifeline EC media (Walkersville, MD) with 2% fetal bovine serum, 15 ng mL^{-1} insulin growth factor, 5 ng mL^{-1} endothelial growth factor, 5 ng mL^{-1} fibroblast growth factor, 50 μg mL^{-1} ascorbic acid, 1 μg mL^{-1} hydrocortisone, 0.75 U mL^{-1} heparin, 10 mM glutamine, and antibiotics. There was no vascular endothelial growth factor (VEGF) in this media. Co-cultured cells were also cultured in this media.

Cell Labeling

Cells were labeled with PKH lipophilic cell membrane label (Sigma-Aldrich, St. Louis, MO). HUVEC cell membranes were labeled green with PKH 67 at 10 mM concentration. OBs were labeled with PHK 26 at 10 mM concentration which labeled the cell membranes red. At this dye concentration, the cell membranes were fluorescent up to 7 days with no evidence of dye transfer between cells.

Scaffold Fabrication

The biphasic composite scaffold consisted of a HA scaffold ring with a PLA scaffold core. These scaffolds were fabricated as follows.

Hydroxyapatite Scaffold Fabrication

The HA scaffold was a ring 10 mm in diameter and 2 mm in height with a 5-mm diameter hole in the center (Fig. 1). This scaffold was fabricated using a polyurethane sponge template with 60 ppi (pores per inch) as previously described by Appleford *et al.*[3] Briefly, the sponge template for this outer shell was first cut to resemble a cylindrical pipe with a hollow core in the middle. This polyurethane sponge template was 2 mm in height, with an outer diameter of 10 mm and an inner diameter of 5 mm, thereby having a 2.5-mm wall thickness. The sponges were then ultrasonically treated

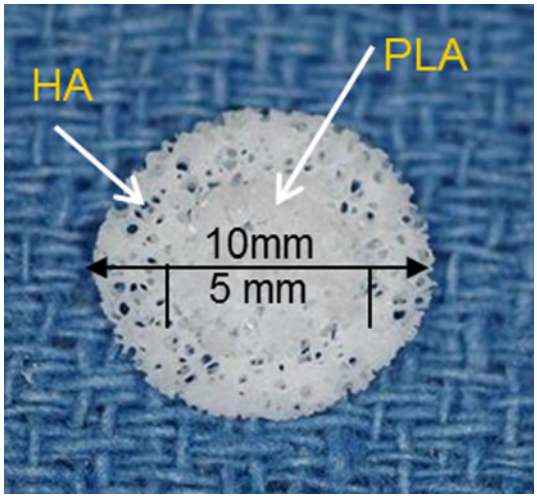

FIGURE 1. Biphasic composite scaffold design with hydroxyapatite ring (HA) and polylactic acid (PLA) core. The diameter of the scaffold is 10 mm and the height is 2 mm.

in 10% sodium hydroxide solution for 20 min, cleaned in flowing water for 40 min, and then rinsed with distilled water. The foam templates were then dried at 80 °C in an oven for 5 h.

Nano-sized HA powder (Berkeley Advanced Biomaterials, Berkeley, CA) was used for fabrication of the scaffold. A 3% (by mass) polyvinyl alcohol solution with 3% (by mass) carboxymethylcellulose, 7% (by mass) of ammonium polyacrylate dispersant, and 5% (by mass) of *N*,*N*-dimethylformamide drying agent was made. HA powder was slowly dispersed into the solution, followed by stirring on low heat until a powder to liquid ratio of 1.50 was obtained. The treated sponge template was immersed in the coating slurry under manual compression until the HA slurry was fully absorbed in the sponge template scaffold. The sponge template was then removed from the slurry and excess slurry removed. The HA slurry-coated sponge template scaffolds was then dried and sintered in a furnace at 1230 °C for 3 h, thus removing the sponge. The scaffolds were sterilized with ethylene oxide. The resulting scaffold had a porosity of 84.4%, pore size of 500 μm, compressive strength of 1.5 MPa, and tensile strength of 60 kPa.[12]

Polylactic Acid Scaffold Fabrication

The 5-mm diameter core of the composite scaffold is a gas–plasma-treated PLA scaffolds fabricated using the vibrating particle salt-leaching method to create an open-cell, interconnected scaffold with porosity >90%, permeability of 24.4 ± 11.3 E 08 m^4 N^{-1} s^{-1}, and pore sizes of 200–600 μm.[1] Briefly, 0.38 g of poly(D,L-lactic) acid polymer (Mw of 109,500 Da, Mn of 64,900 Da, polydispersity of 1.69) (DURECT Corp, Birmingham, AL) was dissolved in 3.25 mL acetone. The polymer solution was added to 5.5 g sodium chloride while being vibrated under continuous air flow conditions in a Teflon mold. 5-mm diameter disks of 2 mm thickness were punched out of the polymer mixture after drying under vacuum and low heat. The sodium chloride was leached out in sterile deionized water. After 2 days in water, scaffolds were lyophilized.[1] GP treatment was performed in a pure oxygen environment in a glow discharge system (PDC-32G, Plasma Cleaner/Sterilizer; Harrick Scientific Inc., New York) for 3 min at 100 W as a surface treatment and for sterilization. The gas–plasma treatment decreases PLA's contact angle from 75° to 30°.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to characterize the surface morphology of the biphasic composite scaffold prior to the seeding of cells. The scaffolds in their entirety were sputter-coated with gold–palladium and imaged using an EVO40 SEM (Zeiss, Germany).

Scaffold Seeding

50,000 cells in 20 μL of media were seeded onto the PLA core, and 150,000 cells in 500 μL of media were seeded onto the HA ring as described below. These cell numbers were chosen to keep the per unit volume cell seeding density consistent between the two scaffolds.

The PLA scaffolds were seeded using the drop-wise method in an ultra-low attachment well plate (Corning, Edison, NJ) after degassing the scaffolds under vacuum. The cells were allowed to attach to the scaffold for 45 min before adding additional media. This method results in 90–95% cell attachment to the PLA scaffold. The HA scaffolds were seeded by incubating the scaffold in a cell suspension of 150,000 cells in 500 μL in an ultralow attachment well plate for 24 h which results in 95–98% cell attachment. After 24 h, there was uniform distribution of the cells on the scaffolds (Fig. 2). After incubating the scaffolds for 24 h separately, the scaffolds were assembled together and incubated for two more days at 37 °C and 5% CO_2 on a shaker plate at a low setting to ensure flow of media throughout the scaffold. On either day 3 or day 5, the scaffolds were removed from the media, rinsed gently with phosphate buffered solution, and fixed with 4% paraformaldehyde.

The scaffolds were seeded in six different seeding arrangements as described in Fig. 3. This allows for six different groups to assess OB and EC migration: four monoculture groups and two co-culture groups. The groups were named X-OB ($n = 6$), X-EC ($n = 6$), OB-X ($n = 9$), EC-X ($n = 6$), OB–EC ($n = 9$), and

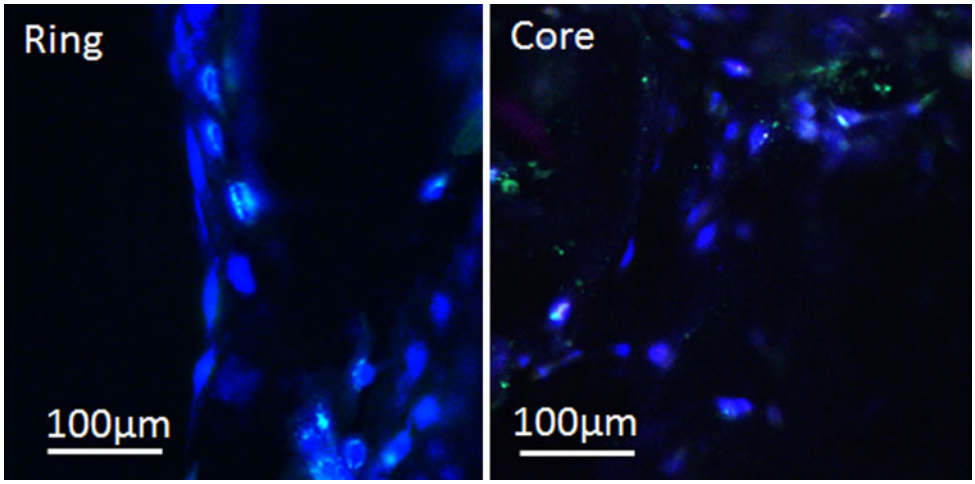

FIGURE 2. Confocal microscopy image of HA ring and PLA core at day 1 showing coverage of scaffold struts with cells (blue—nuclei of EC).

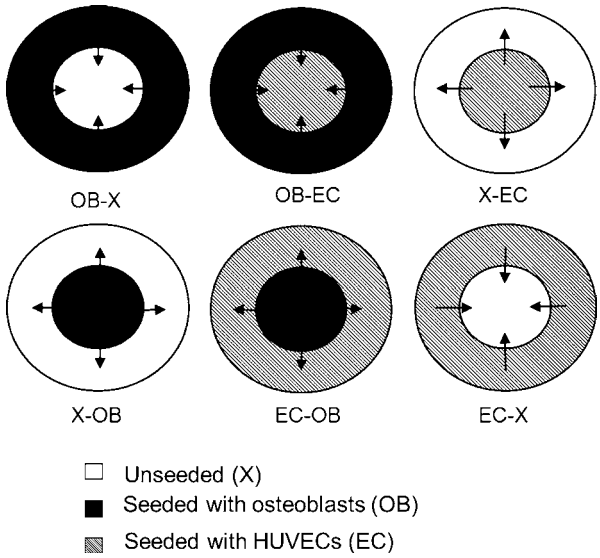

FIGURE 3. Cell seeding diagrams. Shaded areas are seeded with endothelial cells, black areas are seeded with osteoblasts, and blank areas are not seeded with any cells.

EC–OB ($n = 6$). The first part of the designation refers to the type of cell seeded on the HA scaffold phase, and the second part refers to the cell type seeded on the PLA scaffold phase. Areas not seeded with cells were designated "X."

To evaluate OB migration into the PLA core, OBs were seeded on the HA scaffold ring. The core was left unseeded for the monoculture group (OB-X) and seeded with ECs for the co-culture group (OB–EC). Migration of OBs from the PLA scaffold core to the HA ring was assessed by seeding the PLA core with OBs and leaving the ring blank (X-OB) or seeding it with ECs (EC–OB). The co-cultured biphasic scaffolds from the OB migration experiments could also be used for the endothelial migration experiments. EC migration from the HA ring into the PLA core was evaluated by comparing monocultured ECs (EC-X) with co-cultured ECs (EC–OB). EC migration from the core into the ring was evaluated by comparing monocultured ECs in the core (X-EC) with co-cultured core ECs (OB–EC) (Fig. 3).

Imaging and Image Analysis

Prior to imaging, all cell nuclei were counterstained with DAPI. Confocal 3D imaging was performed on the scaffolds with multiple images taken of each phase of the scaffold on days 1 and 3 on a Zeiss 510 LSCM (Zeiss, Germany) at 10×. There were six images per sample with three randomly chosen areas on each phase of the scaffold. The images were reconstructed and analyzed using IMARIS (Bitplane, St. Paul, MN) and the number of cells migrating to the other scaffold phase counted per low power field (LPF) using the spots function. Images of the surface of the scaffolds were also taken using fluorescence microscopy on an inverted fluorescence microscope (Nikon, Melville, NY) on days 1, 3, and 5.

Statistical Analysis

Statistical analysis was performed using Student's t test with significance determined at $p < 0.05$. Results are reported as mean ± standard error.

RESULTS

Scaffold Imaging

SEM images show that the PLA and HA phases of the scaffold were well approximated. The HA and PLA

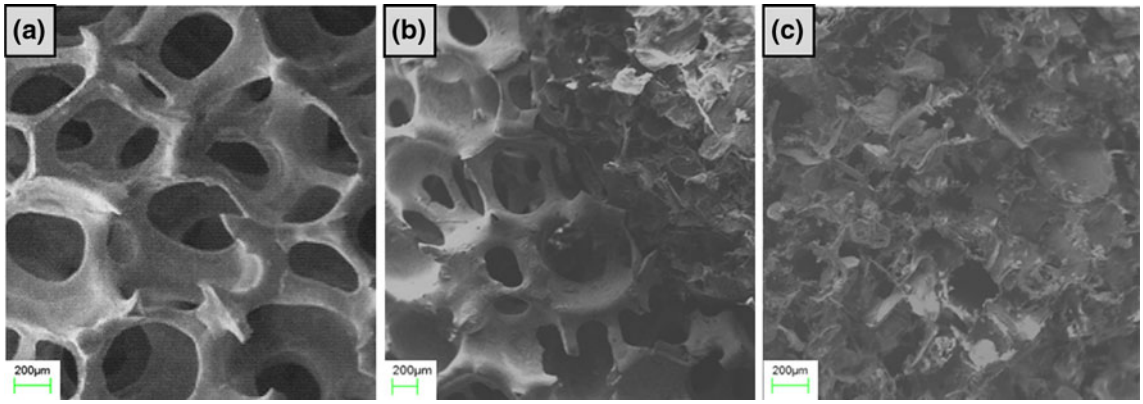

FIGURE 4. Scanning electron microscopy images of (a) HA scaffold, (b) the interface of hydroxyapatite (HA) ring and polylactic acid (PLA) core, and (c) PLA scaffold.

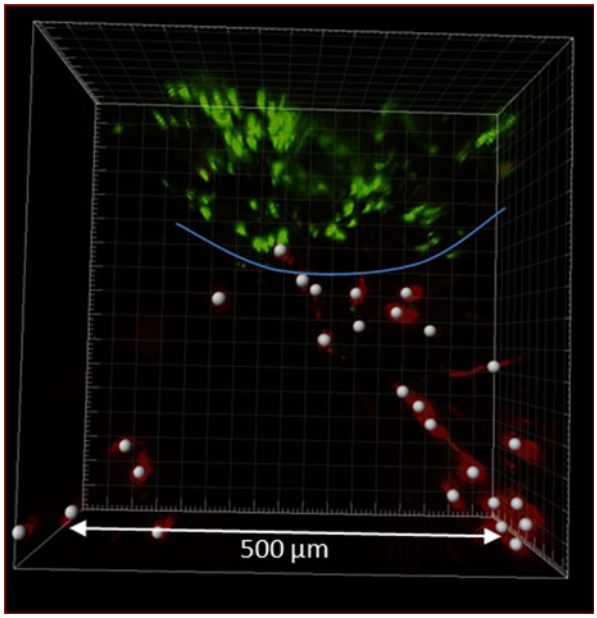

FIGURE 5. Confocal microscopy three-dimensional reconstruction image of the interface between the HA ring and PLA core at day 1 showing no cell migration or contamination during the assembly of the biphasic scaffold. Blue line approximates the actual interface (red and white dots—osteoblasts; green—endothelial cells).

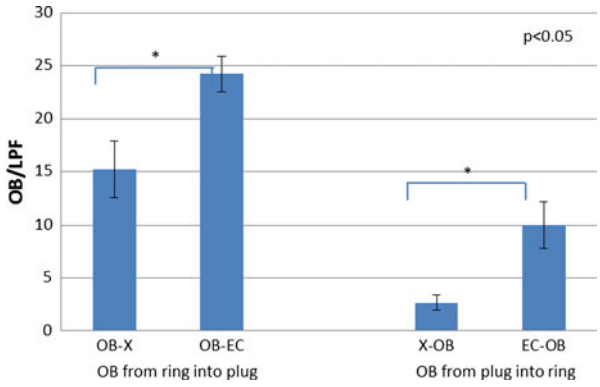

FIGURE 6. Osteoblast migration on day 3, cells/LPF ± SE (n = 6–9, $p < 0.05$). There is a statistically significant increase in migration when osteoblasts are co-cultured with endothelial cells (OB–EC, EC–OB).

scaffolds both have interconnected open pores. The HA scaffolds have struts of 100–200 μm thickness and round pores whereas the PLA scaffolds have square pores with thin walls between each pore (Fig. 4).

Osteoblast Migration

At day 1, which was directly after the two scaffolds were assembled, there was uniform coverage of the scaffold on which the OBs were seeded. No OBs were present in the other section, suggesting that there was no cell migration or contamination during the assembly of the biphasic scaffold (Fig. 5).

At day 3, there was a statistically significant increase in OB migration from one scaffold phase to another ($p < 0.05$) in the OB–EC co-culture group. In monoculture (X-OB), 2.7 ± 0.7 OBs per low power field (LPF) migrated from the PLA core into the HA ring. In contrast, when OBs were co-cultured with ECs (EC–OB), there was an increase of cell migration to 10.0 ± 2.2 OBs per LPF into the HA ring. Similarly, there was a significant increase in OB migration from the HA ring into the PLA core when the cells were co-cultured (OB–EC). In monoculture (OB-X), 15.2 ± 2.7 OBs migrated into the core compared to 24.2 ± 1.7 OBs in co-culture (Fig. 6). Fluorescence and confocal images of the center of the EC-seeded PLA core on day 3 show the presence of OBs, confirming the ability of the OB to migrate into the central areas of the scaffold (Fig. 7). Fluorescence microscopy images were also taken of the scaffolds at day 5. At this time point, it was observed that the OBs and ECs grouped together on HA struts in nodule-like formations (Fig. 8).

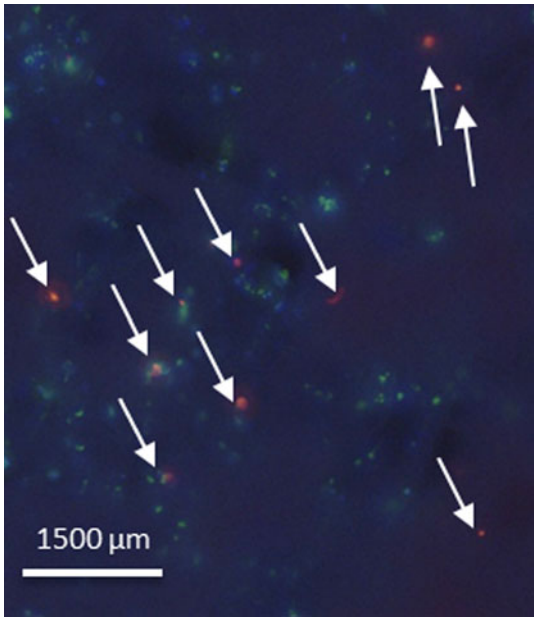

FIGURE 7. Fluorescence microscopy image shows OB (arrows) present in the center of the EC-seeded PLA scaffold on day 3.

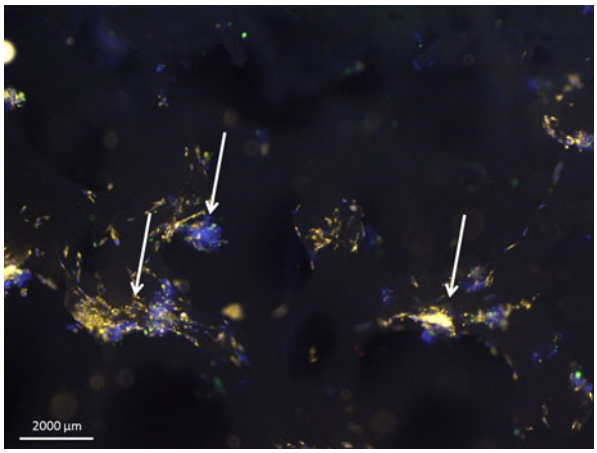

FIGURE 8. Osteoblasts and endothelial cells coming together to form nodules on HA struts on day 5 (arrow).

Endothelial Cell Migration

On day 1, there was uniform coverage of the scaffold surfaces with cells and no migration of EC to the other phases. At day 3, it was apparent that ECs behave differently in co-culture than OBs. ECs in co-culture had a significant decrease in migration from the HA ring into the PLA core. In monoculture (EC-X), the number of cells migrating into the core per field was 74.4 ± 19.2 cells per LPF, but when in co-culture (EC-OB), the number of migrating cells from the ring into the core per LPF decreased to 33.0 ± 7.6 cells per LPF.

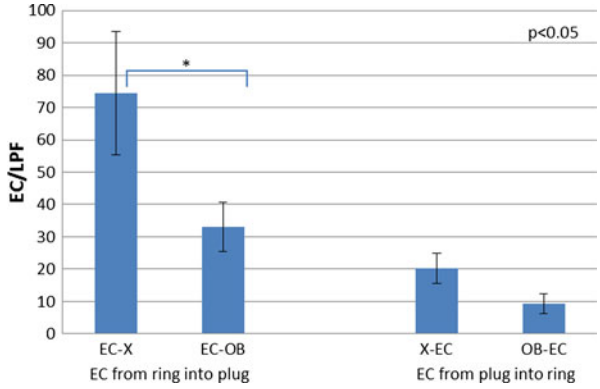

FIGURE 9. Endothelial cell migration on day 3, cells/LPF ± SE ($n = 6–9$, $p < 0.05$). Endothelial cell migration decreased when co-cultured with osteoblasts (EC–OB).

Endothelial cell migration from the PLA core into the HA ring was also decreased when the cells were co-cultured (OB–EC), but this decrease was not statistically significant ($p = 0.06$). EC migration into the ring was 20.3 ± 4.6 cells per LPF in monoculture (X-EC) and 9.3 ± 1.7 cells per LPF in co-culture (OB–EC) ($p < 0.05$) (Fig. 9).

DISCUSSION

Osteoblast–Endothelial Cell Interactions

This is the first study that observes the migration patterns of OB and ECs in co-culture within a biphasic tissue-engineering scaffold system. Most co-culture studies investigating migration are not on three-dimensional scaffolds or are in models such as spheroids and Boyden chambers. These models do not necessarily replicate the environment to which the cells will be exposed inside a tissue-engineered scaffold. In this study, EC and OB co-cultures in a biphasic composite scaffold resulted in increased OB migration and decreased EC migration. When comparing these results to natural bone healing, it is possible to explain these changes in cell migration patterns.

In natural bone healing, a vascular network is setup prior to and during the influx of osteogenic cells.[4] The presence of a critical number of ECs is necessary before vessel formation, and the establishment of a vascular network can begin. However, the presence of ECs by themselves is not enough to start the development of a mature vascular network. Multiple studies have shown that in monoculture, ECs form tortuous, immature capillary-like structures.[14,18] In order for these vessel structures to be stabilized, it is necessary to have mural cells.[18] In the presence of mural cells, the ECs begin to slow their movement and line up in vessel-like formations.[13] The limitation of EC migration may have an important role

in angiogenesis because ECs need to stay localized to form tube-like structures. Other studies have also shown that in the presence of OBs, a decrease in EC migration occurs.[31] This phenomenon is in concordance with the present study, where it was noted that ECs did not migrate to the same extent in co-culture with OBs as they did in monoculture. The exact mechanism of this occurrence is unknown but it is thought to be due to VEGF, a known potent angiogenic factor,[25,30] as well as many other growth factors secreted by OBs.

Stahl *et al.*[27] showed that when EC and OB are co-cultured together, senescence of the ECs is induced. This effect was seen in the co-culture EC–OB configuration. There was a significant decrease in EC migration compared to the ECs in the EC-X configuration. The OB–EC configuration showed a trend of decreased migration of ECs, but it did not reach levels of significance, mostly due to the variability in the cell numbers. The variability is present because there were more migrating cells present in the scaffold areas closer to the opposing scaffold phase than at the areas further away, and the areas imaged on each scaffold were randomly chosen.

Once the vascular network is formed, osteogenic cells start to form immature bone in natural bone healing. For bone formation to occur successfully, it is necessary for the framework of the vascular network to be in place and continue to expand.[4] In this study, OB migration was increased when the cells were in the presence of ECs, reflecting the increased likelihood of bone formation in co-culture. Additionally, when cells were co-cultured on HA struts, grouping of the cells reminiscent of mineralization nodules was observed on day 5 (Fig. 8).

The formation of bone in EC and OB co-cultures has not been studied as extensively as the formation of vasculature in these co-cultures. However, the effects of ECs on the bone forming capabilities of OBs must also be studied in the co-culture systems since it is evident there is a type of communication occurring between the ECs and OBs.[7,24] It has been shown in migration studies that these cells have a crosstalk that influences the ECs to increase angiogenesis and OBs to increase osteogenesis while inducing senescence of ECs.[27] These effects are thought to be due to the gap junctions between the EC and OB since these effects are only seen when the cells are touching each other.[29] Gene expression analysis of HUVEC and OB co-cultures has shown changes in many EC genes, most notably an up-regulation of platelet-derived growth factor receptor-α and vascular endothelial growth factor receptor-2.[10]

Scaffold Design

For bone regeneration in this scaffold design, OBs are to be seeded on the outside on the HA ring and ECs on the inner PLA core. The purpose of this seeding strategy is to promote bone formation on the outside and vessel formation in the inside.

When developing the biphasic composite scaffold, it was important to determine if cells could cross the different material boundaries. The results of this study indicated that cells are not only able to migrate from one phase to another, but are also capable of migrating into areas seeded with other cell types. As evidenced at day 1, both cells types were able to attach and spread out on the struts of the two different scaffolds.

Hydroxyapatite is a favorable hydrophilic surface for OB attachment.[20] PLA surfaces are relatively hydrophobic in comparison to HA and are often modified in order to improve cell attachment and proliferation. One form of surface modification process is the oxygen gas–plasma treatment used in this study. Studies with oxygen gas–plasma-treated PLA show increased hydrophilicity of the surface to contact angles of 30° and increased oxygen functional groups on the surface. These alterations have been reported to improve cell attachment and proliferation on the polymer surface.[5,26]

When OBs are cultured *in vitro* with ECs, increased OB filopodia have been reported, in addition to the increased aggregation of the cells into nodule-like formations.[31] This finding is in agreement with the day 5 observations of this study. These observations support the hypothesis that there is increased OB migration when they are co-cultured with ECs.

The fact that the ECs and OBs will migrate both into the central areas and the periphery of the scaffold is counter-intuitive since central areas of the scaffold are likely to have a lower oxygen content than the periphery, with a significant decrease in oxygen tension inside a scaffold after a depth of 200 μm.[15] In our study, there was evidence of migration into the central areas of the PLA scaffold even 500 μm deep into the scaffold and 5 mm from the edge of the HA ring based on confocal images. However, this migration pattern may not be seen in scaffolds larger than 1 cm since diffusion of oxygen and nutrients will be more limited due to the larger size. This culture system was placed on a plate shaker to improve flow throughout the scaffold which would increase the amount of oxygen and nutrients going into the central areas while removing by-products.

The results of this study also indicate that the OBs do not show preferential migration based on the scaffold material since there was an increase in migration whether it was seeded on the PLA core or HA ring. The overall differences in cell numbers of OBs and ECs migrating from the PLA core to the HA ring vs. from the HA ring into the PLA core can be accounted for by the differences in cell seeding numbers. The HA ring was seeded with three times as many cells as the PLA core.

Studies have shown that the cells behave differently when cultured together in direct contact, where cells physically touch each other, vs. in indirect contact, where cells are in the same culture well but are not able to form physical contacts. Direct contact allows the ECs and OBs to form gap junctions via connexin 43 and the cell membrane proteins to interact. As a result of these juxtacrine interactions, an increase in the amount of alkaline phosphatase and collagen I have been reported.[29] In this scaffold system, the cells were seeded on different phases and then allowed to migrate and interact with each other. Thus, this system resulted in a hybrid "indirect" and "direct" co-culture. All cells are exposed to the secreted cytokines and signals while the migrating cells can form direct contacts with the other cell type they encounter.

CONCLUSION

In this study, the use of a biphasic composite scaffold allowed for cell migration between the HA and PLA phases of the scaffold. There was increased OB migration into and out of the central areas of the scaffold when they were co-cultured with ECs. In contrast, EC migration was lower when they were co-cultured with OBs. It was thus concluded from this study that a way to improve OB migration into the interior of scaffolds is to seed ECs into central areas of the scaffold.

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